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SINGLE PHOTON PHASE METHOD FOR RADIATIVE LIFETIMES AND POLARIZATION ANISOTROPY DECAY

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ABSTRACT: The measurement of fluorescent lifetimes and of polarization anisotropy decay is a powerful method for probing biological systems due to the combination of high sensitivity, high time resolution (vide infra) and the ease with which fluorophores may be found in (or inserted into) biological systems. The Single-Photon Phase Method is a hybrid of two methods which are, respectively, the phase-shift method and the single photon time correlation method. It offers advantages in both sensitivity and time resolution. 2 picosecond differences have been measured.

Since the method I am reporting is hybrid, it is most easily explained in terms of its parents. The two main approaches to fluorescence lifetimes are impulse (flash-lamp) and modulation-transfer (continuous, periodic excitation). Formally the approaches are equivalent (Eigen,M. & de Maeyer,L. 1963). Experimentally they differ widely. Both will briefly be described here. Phase shift methods were developed early, and have undergone continual improvement (Bailey,E.A. & Rollefson,G.K. 1953; Spencer,R.D. & Weber,G. 1969) A sinusoidal intensity function is applied on the excitation side of the sample, and the phase-shift (and/or modulation index) is compared on the emission side. The circuitry for the phase meter is

the most critical and difficult part of such apparatus. This is particularly so if emission is very weak, since it is then necessary to operate with very low level analog signals. In Weber's design, the comparison signal is mixed in the photomultiplier. In order for the apparatus to be optimally sensitive, the frequency should be variable, since the phase shift goes as the arctan of the product of the angular frequency and the lifetime. In addition, in the case of systems with multiple decay processes, it is necessary to sweep the frequency. It is often erroneously stated that impulse methods give better results with multiple decay systems; while it is true that a visual indication is given with relatively little pain, quantitative data, particularly in the case of comparable relaxation times, must be extracted by a computation that is no less difficult than that involved in the phase method. Pulse methods evolved from simple monitoring of decay curves to what is usually called the 'single-photon' method (Ware,W.R. 1969) In this technique, the excitation must be of sufficiently low intensity that at most one detectable photon is emitted after each pulse. Usually in cases of interest emission intensity is too low anyway due to sample problems and lamp intensities. The time of arrival of the emitted photon is measured with respect to the excitation time by taking a signal from the pulse lamp or the lamp circuit as a reference, and using this as the start signal for a time-to-amplitude converter (TAC). The stop signal is taken from the emission photomultiplier (in counting mode). The TAC generates a linear ramp, so that output voltage now is equivalent to time and this signal

is converted to a histogram of number of pulses (photons) versus time. While rather round-about, this proceedure offers considerable advantage over simpler methods -principally in the area of time resolution. Unfortunately it is critically dependent on flash lamps with very short rise times, and thus two problems arise: first of all such sources are weak and have a low duty cycle (this may be avoided by use of pulse lasers,at the cost of fixing the wavelength range available) and secondly, the shape of the excitation function is not known (in general). For lifetimes within the order of magnitude of the lamp decay time, rigorous deconvolution is not possible.

The Single-Photon Phase Method combines features of both, in order to achieve higher sensitivity (i.e. capability to work with very weak emission (Bender,R.,Jankow,R.,& Renken,W. 1970)) and better time resolution. An additional advantage is the easy addition of strobing to use the system to follow changes in rotational relaxation as a function of time. Keeping the two methods described above in mind,the technique is as follows: the continuous source is intensity modulated the same way as for the standard phase method. However, a zero-crossing discriminator, driven by the modulator circuit, produces a series of timing pulses (one pulse per cycle). Now, analogous to the single-photon technique, there exists the requirement that there be no more than one emitted photon per cycle. Detected photons give rise to pulses which are processed in circuitry identical to that used in the single-photon experiments. The timing signal and the

emission signal are now used by a TAC to generate a voltage proportional to time of arrival of the photon relative to a fixed point in the cycle (not necessarily the beginning). A histogram of these times gives one cycle of the input function after modulation by the sample (plus a uniform background); a scattering sample is used for a reference. A stable oscillator allows long runs with very weak emitters. With a continuous source, under normal circumstances, far more data can be acquired in a reasonable time than with the single-photon method, due to higher excitation flux. This allows averaging out of electronic timing errors. With conventional, off-the-shelf circuitry 2 picosecond time differences have been measured. Since the signal is immediately converted to digital data,(at the PMT in pulse-mode), the signal to noise ratio is limited by the photocathode. It should be remarked, in conclusion that due to the large number of common elements it is very easy to upgrade existing 'single-photon' apparatus to this new method.

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